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Virucidal Treatment of Blood Protein Products with UVC Radiation

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ABSTRACT

The virus safety of blood derivatives continues to be of concern, especially with respect to nonenveloped and/or heat-stable viruses. Previously, we demonstrated that treatment of whole plasma, AHF concentrate or fibrinogen with short wavelength ultraviolet light (UVC) results in the inactivation of $\geq 10^6$ infectious doses (ID) of encephalomyocarditis virus (EMCV), hepatitis A virus (HAV) and porcine parvovirus (PPV), each of which is nonenveloped. Protein recovery was enhanced greatly by inclusion of the flavonoid, rutin, added prior to UVC exposure to quench reactive oxygen species. We now report on the treatment of albumin and intravenous immune globulin (IVIG) isolated by a previously described, integrated chromatographic method. Albumin was treated with either 0.1 or 0.2 J/cm^2 UVC in the presence of 0.8 or 1.6 m*M* rutin; IVIG was treated with either 0.05 or 0.1 J/cm^2 UVC in the presence of 0.5 or 1.0 m*M* rutin. Our results show that $\geq 10^{6.9}$ ID of EMCV and PPV were inactivated under each of the conditions studied except the treatment of albumin with 0.1 J/cm^2 UVC in the presence of 1.6 m*M* rutin where $10^{4.3}$ ID of EMCV and $\geq 10^{6.9}$ ID of PPV were killed. It appears that the sensitivity of PPV to UVC exceeds that of EMCV and that virus kill with UVC is higher in IVIG than in albumin. In the absence of rutin, UVC increased the extent of aggregation of both albumin and IVIG by two- to three-fold. With rutin present, the increase in albumin aggregation was reduced, and it was virtually eliminated by subsequent processing on Sephacryl S-200, a step in the existing procedure designed to remove aggregates. The increase in aggregation of IVIG appeared to be eliminated on inclusion of either 0.5 m*M* or 1 m*M* rutin. We conclude that both albumin and IVIG can be treated with UVC to inactivate $\geq 10^6$ ID of nonenveloped viruses. The inclusion of rutin during treatment helps protect against protein aggregation.

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INTRODUCTION

The transmission of hepatitis A virus (HAV)† and parvovirus B 19 by the current generation of virally inactivated blood protein products has raised concern about nonenveloped viruses present in these products (1–5). This concern is heightened by the observation that nonenveloped viruses are more resistant than enveloped viruses to currently employed virucidal procedures, including solvent/detergent and heat treatments (6,7). Because both HAV and parvovirus have single-stranded genomes, both should be inactivated readily by short wavelength (254 nm) ultraviolet light (UVC). This report extends our previous studies with AHF, fibrinogen and plasma (8,9) to intravenous immune globulin (IVIG) and albumin.

MATERIALS AND METHODS

Protein preparations

Albumin (6%) and IVIG (5%) solutions were prepared chromatographically. Briefly, plasma was desalted on Sephadex® G-25-C as outlined in Fig. 1. After removal of euglobulins by precipitation, the plasma was applied onto DEAE Sepharose® FF. The IVIG was present in the passthrough fraction while albumin bound to the resin. Following elution, albumin was further purified on CM Sepharose FF, treated at 55°C for 3 h to remove lipids, UVC treated and then passed through Sephacryl® S-200 HR. The IgG fraction was purified on Q Sepharose FF and CM Sepharose FF, concentrated, UVC treated and then passed through CM Sepharose FF.

Virus inactivation

Before irradiation, the indicated virus and quencher were added to the protein solution under study, after which the mixture was pumped peristaltically through a flat quartz cell (15 cm × 1.2 cm × 0.22 mm internal dimensions, 0.4 mL volume). The mixtures were irradiated using a low-pressure mercury lamp (General Electric no. BLEIT155; Spectronics Corp., Westbury, NY) emitting 90% of its

†*Abbreviations:* AHF, antihemophilic factor concentrate; DMEM, Dulbecco's modified Eagle's medium; EMCV, encephalomyocarditis virus; FBS, fetal bovine serum; FPLC, fast flow liquid chromatography; HAV, hepatitis A virus; ID, infectious doses; IVIG, intravenous immune globulin; PBS, phosphate-buffered saline; PPV, porcine parvovirus; RIFA, radioimmunofocus assays; ROS, reactive oxygen species; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; UVC, short wavelength (254 nm) UV light.

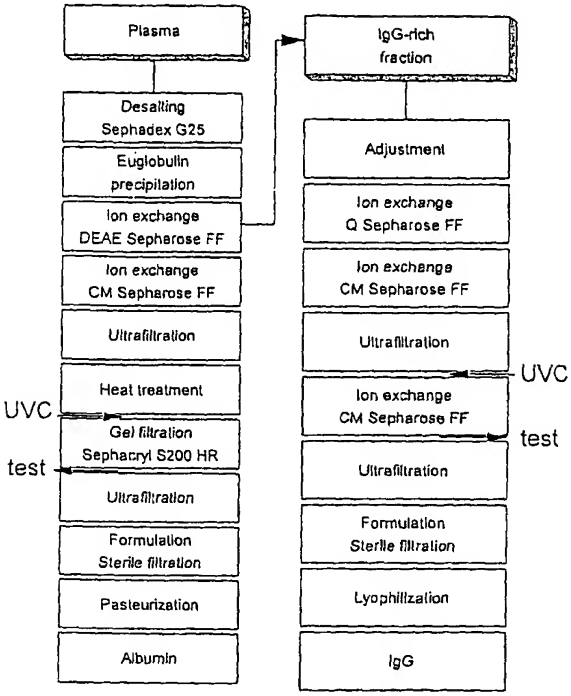

Figure 1. Chromatographic purification of albumin and IgG. Albumin and IgG were purified by procedures established by Pharmacia Biotech AB, as outlined in this figure. The UVC treatment occurred at the purification stage indicated. Virus was added just prior to, and viral infectivity determined just following UVC treatment. Protein integrity was assessed at the same stage and also following the subsequent purification step. At the time of UVC treatment, albumin was at 60 mg/mL and IgG was at 50 mg/mL total protein.

energy at 254 nm. Irradiance was measured with a Spectroline DM-254H Digital Radiometer (Spectronics Corp.), and total radiant energy was controlled by pump rate. A fluence of 0.1 J/cm^2 corresponded to a pump rate of 2 mL/min and an exposure time of approximately 12 s.

Viruses and viral assays

Studies on the inactivation of encephalomyocarditis virus (EMCV) and HAV were performed as described previously (10–12). For EMCV, we assessed infectivity by endpoint, 10-fold serial dilutions using each dilution to inoculate eight replicate wells containing Vero cells (American Type Culture Collection, Rockville, MD) in 96 well microtiter plates. Virus-induced cytopathology was scored after 72 h of incubation at 37°C in 5% CO_2. Viral titer, calculated by the Spearman–Karber method (13), indicates the quantity of virus that infects 50% of the tissue culture wells.

The HAV strain HM175/18f was obtained from Dr. Stanley M. Lemon (University of North Carolina, Chapel Hill). Infectivity titrations were determined by radioimmunofocus assays (RIFA) carried out in BS-C-1 cells, also obtained from Dr. Lemon (14). To determine the titer of HAV in the samples, 10-fold serial dilutions were made between 10^1 and 10^6 in Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, NY) containing 5% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD). Each dilution was tested in singlet for infectious virus by inoculating BS-C-1 cells in petri dishes with 0.25 mL of the sample, incubating for 2 h, after which the cells were overlaid with 0.5% agarose containing DMEM and 2% FBS. After culturing at 37°C in 5% CO_2 for 6 days, the overlay was removed, the cells were fixed with acetone for 10 min and reacted with ^{125}I-anti-HAV antibody (HAVAB kit, Abbott Laboratories, Chicago, IL) diluted 20-fold with phosphate-buffered saline (PBS) containing 10% FBS at 37°C for 4 h. Excess antibody was removed by washing three times with PBS containing 0.02% sodium azide and the foci visualized by radioautography on Kodak Scientific Imaging Film X-OMAT AR (Kodak, Rochester, NY).

Porcine parvovirus (PPV) and parvovirus infectivity assays were conducted by Dr. E. Dubovi (Cornell University, Ithaca, NY). Ten-fold serial dilutions (0.05 mL) of treated samples were added to an established line of swine testicle cells in 16 well Lab Tek plates with each dilution inoculated into eight wells. The inoculum was allowed to adsorb for 2 h and was then removed and medium added. After a 3–4 day incubation at 37°C, the cells were washed, fixed in acetone and stained for the presence of PPV with a monoclonal anti-parvovirus antibody as the primary antibody and fluorescein-conjugated, goat anti-mouse antibody as the secondary antibody.

Table 1. The UVC treatment of AHF, plasma and fibrinogen*

Solution	Rutin	Protein recovery (%)	Virus kill (log_{10}) HAV	PPV
AHF	+	80–90	≥5.3	≥5.5
	−	20	≥5.3	≥5.5
Plasma	+	60–90	≥5.3	≥6.0
	−		≥5.3	≥6.0
Fibrinogen	+	100	≥5.1	≥5.5
	−	≤50	≥5.1	≥5.5

*The solutions were treated with 0.1 J/cm^2 UVC in the presence or absence of rutin, added at a concentration of either 0.5 m*M* (AHF, fibrinogen) or 0.8 m*M* (plasma). Following treatment, coagulation factor activity and the infectivity of virus were assessed.

Protein analysis

Electrophoresis. Native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed using 8 cm × 8 cm × 1.5 mm precast 4–12% gradient gels for plasma and 6% gels for AHF concentrates (Novex, San Diego, CA). The gels were run with Tris-glycine native buffer or Tris-glycine-SDS running buffer (Novex) on the Novex Xcell Mini-Cell at 125 V for 120 min. Staining was with Coomassie brilliant blue (BioRad Laboratories, Melville, NY).

Protein aggregation. Fast flow liquid chromatography (FPLC) analysis was performed on Superdex® 200. Samples of 200 μL were applied onto 60 mL resin at a flow rate of 0.5 mL/min and eluted with 150 m*M* NaCl + 50 m*M* phosphate, pH 7. Full scale deflection was set at 0.5 A_{280}.

RESULTS

The UVC treatment of AHF concentrate, whole plasma and fibrinogen results in the complete inactivation (≥$10^{5.3}$ to ≥$10^{6.0}$ infectious doses [ID]) of HAV and PPV (Table 1). Inclusion of the flavonoid rutin significantly improved protein functional recovery without depressing virus kill significantly (Table 1). We therefore studied the effect of UVC treatment on albumin and IVIG.

Albumin and IVIG

Albumin and immune globulin have been traditionally prepared using cold ethanol precipitation as the principal purification procedure. However, newer chromatographic procedures, such as the one described in Fig. 1, have several

Table 2. The UVC treatment of chromatographically purified albumin and IVIG*

Solution	UVC (J/cm²)	Rutin (m*M*)	Virus inactivation EMCV	(Log_{10}) PPV
Albumin	0.10	0.8	6.1	≥6.9
	0.10	1.6	4.3	≥6.9
	0.20	0.8	>6.3	≥6.9
	0.20	1.6	6.2	≥6.9
IVIG	0.05	0.5	≥5.8	≥6.6
	0.05	1.0	≥5.8	≥6.6
	0.10	0.5	≥5.8	≥6.6
	0.10	1.5	≥5.8	≥6.6

*At the steps indicated in Fig. 1, EMCV or PPV, together with rutin at the concentrations indicated, were added to purified albumin and IgG, after which the solutions were exposed to UVC. Virus inactivation was assessed without further purification. Protein integrity was assessed as described in the text.

advantages including ease of implementation, higher protein recovery and improved flexibility in meeting throughput requirements. In implementing a new purification system, questions of viral safety inevitably arise, thus favoring inclusion of an additional virus inactivation step. We evaluated UVC treatments at the steps indicated (Fig. 1); viral results are reported in Table 2. Albumin at 60 mg/mL was treated at 0.1 and 0.2 J/cm² UVC with or without rutin. At least 10^6 ID of nonenveloped viruses were inactivated with 0.1 J/cm² in the presence of 0.8 m*M* rutin. Increasing the rutin concentration to 1.6 m*M* suppressed virus kill somewhat, unless UVC fluence was also increased. The sensitivity of parvovirus to UVC treatment must be very high because complete kill was achieved under all conditions studied, including a UVC fluence as low as 0.05 J/cm². Without irradiation, a small quantity of albumin dimer was evidenced, both on Superdex 200 chromatography and on SDS-PAGE (Fig. 2). Normal processing through Sephacryl S-200HR removed dimers and larger aggregates. For treatment of 6% albumin, 0.1 J/cm² UVC in the presence of 0.8 m*M* rutin appeared to be optimal.

The IVIG at 50 mg/mL was treated at 0.05 and 0.1 J/cm² UVC in the presence of 0–0.1 m*M* rutin. Complete kill ($\geq 10^{5.8}$ to $\geq 10^{6.6}$) of added EMCV and PPV was observed at both UVC fluences regardless of the concentration of added rutin. Even at the higher UVC fluence (0.1 J/cm²), little increase in aggregate content was observed by FPLC (Fig. 3, left panel). In contrast, SDS-PAGE (Fig. 3, right panel) or FPLC analysis following CM-Sepharose FF (Fig. 3, middle panel, the next purification step) revealed a UVC dose-dependent increase in aggregate content that was reduced substantially by rutin presence.

DISCUSSION

New methods for the inactivation of nonenveloped viruses present in blood protein preparations need to be developed. It has long been known that nucleic acids are more sensitive than proteins to UVC, principally because nucleic acids absorb more energy than proteins at light wavelengths between 240 and 260 nm and because of their larger target size. Nonetheless, protein damage has limited the dose of UVC to levels that were too low to ensure safety. Based on our observations that rutin protects proteins without affecting virus kill, it would appear that a significant amount of protein damage is mediated by reactive oxygen species (ROS) while virtually all virus kill results from the direct absorption of photonic energy. We have now evaluated application of this approach to a variety of blood proteins and blood protein products, including AHF concentrates, plasma, fibrinogen, albumin and IVIG. In each case, regardless of the concentration of protein, $\geq 10^6$ ID of HAV (or EMCV, a member of the same virus family) and parvovirus were inactivated on exposure to 0.05–0.1 J/cm² UVC. Protein recovery in most cases exceeded 80%, although on treatment of plasma, the recovery of some coagulation factors was only 60%. Based on these and other supportive data, the U.S. Food and Drug Administration has approved clinical investigations of a UVC-treated fibrin sealant.

In general, we conclude that UVC treatment inactivates substantial quantities ($>10^5$ infectious doses) of both enveloped and nonenveloped viruses. Combination of UVC with established virucidal procedures, such as solvent/detergent treatment will substantially enhance the probability of safety of blood protein products or other products where virus presence is a concern. The specificity of UVC in killing virus without affecting proteins is improved by quenching ROS through rutin addition.

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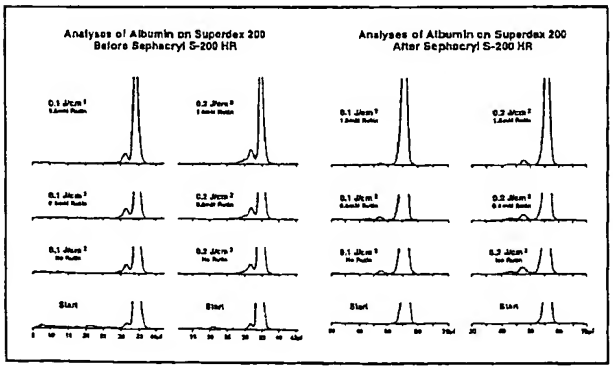


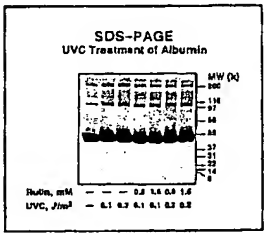
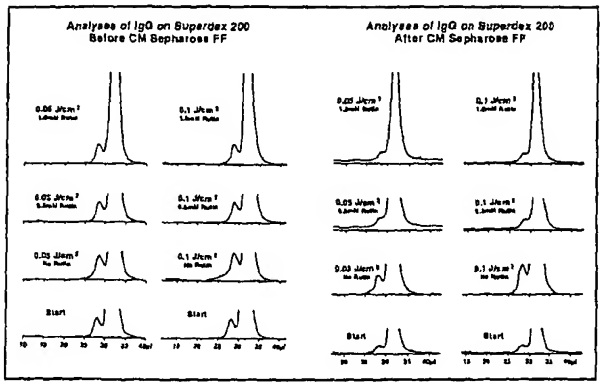


Figure 2. Protein analysis of UVC-treated albumin. Albumin solution was treated at the step indicated in Fig. 1 using UVC and rutin at the conditions indicated in Fig. 2. Albumin aggregation was assessed by FPLC on Superdex 200 (left panel) and by SDS-PAGE (right panel).



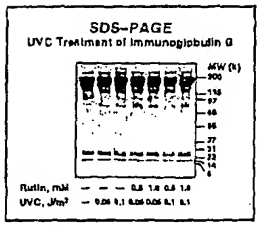


Figure 3. Protein analysis of UVC-treated IgG. The IgG solution was tested at the step indicated in Fig. 1 using UVC and rutin at the conditions indicated in Fig. 3. The IgG aggregation was assessed by FPLC on Superdex 200 (left panel) and by SDS-PAGE (right panel).

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